APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1998, p. 3166–3174 Vol. 64, No. 9
0099-2240/98/$04.00+0

Analysis of *Escherichia coli* O157:H7 Survival in Ovine or Bovine Manure and Manure Slurry

INDIRA T. KUDVA, KATHRYN BLANCH, AND CAROLYN J. HOVDE*

Department of Microbiology, Molecular Biology, and Biochemistry, University of Idaho, Moscow, Idaho 83844

Received 29 April 1998/Accepted 11 June 1998

Farm animal manure or manure slurry may disseminate, transmit, or propagate *Escherichia coli* O157:H7. In this study, the survival and growth of *E. coli* O157:H7 in ovine or bovine feces under various experimental and environmental conditions were determined. A manure pile collected from experimentally inoculated sheep was incubated outside under fluctuating environmental conditions. *E. coli* O157:H7 survived in the manure for 21 months, and the concentrations of bacteria recovered ranged from $<10^2$ to 10^6 CFU/g at different times over the course of the experiment. The DNA fingerprints of *E. coli* O157:H7 isolated at month 1 and month 12 were identical or very similar. A second *E. coli* O157:H7-positive ovine manure pile, which was periodically aerated by mixing, remained culture positive for 4 months. An *E. coli* O157:H7-positive bovine manure pile was culture positive for 47 days. In the laboratory, *E. coli* O157:H7 was inoculated into feces, untreated slurry, or treated slurry and incubated at −20, 4, 23, 37, 45, and 70°C. *E. coli* O157:H7 survived best in manure incubated without aeration at temperatures below 23°C, but it usually survived for shorter periods of time than it survived in manure held in the environment. The bacterium survived at least 100 days in bovine manure frozen at −20°C or in ovine manure incubated at 4 or 10°C for 100 days, but under all other conditions the length of time that it survived ranged from 24 h to 40 days. In addition, we found that the Shiga toxin type 1 and 2 genes in *E. coli* O157:H7 had little or no influence on bacterial survival in manure or manure slurry. The long-term survival of *E. coli* O157:H7 in manure emphasizes the need for appropriate farm waste management to curtail environmental spread of this bacterium. This study also highlights the difficulties in extrapolating laboratory data to on-farm conditions.

Enterohemorrhagic *Escherichia coli* O157:H7 was first identified as a human pathogen in 1982 (47, 53) and since 1982 has been implicated in numerous outbreaks of hemorrhagic colitis and the life-threatening hemolytic uremic syndrome (1, 19, 20, 29). Multiple factors contribute to the pathogenicity of this serotype; these factors include the production of Shiga toxin type 1 and/or type 2, the *eae* genes, and a 60-MDa plasmid encoding adhesins and hemolysins (8, 19).

Healthy cattle and sheep sporadically harbor *E. coli* O157:H7 in their gastrointestinal tracts (GIT) and shed the bacteria in their feces (12, 22, 34, 35). In cattle, *E. coli* O157:H7 occurs with an overall prevalence of 0.3 to 6.1%, and the average length of time that feces from an individual animal remain culture positive is 30 days (49, 58, 63). Culture-positive sheep occur at a rate of 0.9 to 31%, and experimentally inoculated sheep shed the organism for up to 50 days (34, 35, 57). In addition, animals can remain intermittently culture positive for more than 1 year (34, 65). Thus, cattle and sheep are reservoirs of this human pathogen. Other animals from which *E. coli* O157:H7 has been isolated include deer, horses, dogs, and birds (30, 46, 58, 59). Feces from any of these animals could serve as the primary source for *E. coli* O157:H7 contamination of various food products.

Most *E. coli* O157:H7 outbreaks are linked to the consumption of contaminated, undercooked, bovine food products (8, 15, 19). Other sources of infection include contaminated, unpasteurized apple cider, water (drinking and swimming), vegetables, mayonnaise, delicatessen food, lamb, venison, deer jerky, cured salami, and direct contact (animal to person or person to person) (4, 9, 30, 52, 53). Contamination of nonruminant food sources of infection is most often from ruminant manure (48, 53, 61). For instance, apple ciders implicated in a 1991 Massachusetts outbreak and 1996 multistate outbreak were found to be contaminated with cattle manure and deer manure, respectively (6, 10, 11, 30). Likewise, vegetables associated with several outbreaks since 1992 were found to have been grown in soil layered with manure (58). In 1993, Cieslak et al. isolated *E. coli* O157:H7 from a manure-treated garden that was the source of infection-causing vegetables (13). Fecal contamination of meat at slaughter plants and subsequent cross-contamination of other food products at retail shops are another implicated source of contaminated foods (4, 53). In addition, direct contact with bovine or ovine feces has also been associated with *E. coli* O157:H7 infections on farms (3, 56, 61).

Effluents from farming operations include raw manure, untreated slurry (a mixture of manure, urine, split feed, and water that is held without aeration), and treated slurry (the retentate) or aerated slurry that is filtered to separate the solid fraction from the liquid fraction) (26, 64). These effluents are often applied as fertilizer to land used for silage, grazing, or cultivation (26, 40, 64). Unless appropriately processed, manure is a potential biohazard capable of transmitting infective agents, including *E. coli* O157:H7, to both humans and animals (26, 50, 55, 64). Studies have shown that a variety of conditions can influence the survival of pathogenic bacteria (salmonellae, *Mycobacterium paratuberculosis*) and viruses (pseudorabies virus, porcine reproductive and respiratory syndrome viruses, rotaviruses, herpesvirus) that subsequently infect livestock (2, 14, 17, 18, 26, 43, 51, 64). These conditions include temperature, solid content, pH, bacterial concentration, aeration, and the length of time that manure or slurry is held before it is applied to pastureland. Wang et al. (60) demonstrated that the

* Corresponding author. Mailing address: Department of Microbiology, Molecular Biology, and Biochemistry, University of Idaho, Moscow, ID 83843. Phone: (208) 885-5906. Fax: (208) 885-6518. E-mail: cbohach@uidaho.edu.

survival rates of *E. coli* O157:H7 in bovine feces varied depending on temperature and the initial bacterial inoculum. These authors found that *E. coli* O157:H7 survived for the longest time (70 days) at 5°C when it was inoculated at a rate of 10^5 CFU/g of feces (60).

In this investigation we analyzed the survival of *E. coli* O157:H7 (i) in sheep manure and cattle manure exposed to fluctuating environmental conditions and (ii) in experimentally inoculated manure, untreated slurry, and treated slurry incubated under different laboratory conditions. In addition, the role of Shiga toxin type 1 and 2 genes in *E. coli* O157:H7 survival in bovine manure or manure slurry was assessed. Since Shiga toxin is cytotoxic to some eukaryotic cells (21, 31, 37), its presence may provide a survival advantage to *E. coli* O157:H7.

MATERIALS AND METHODS

Manure in the environment. Feces from ruminant animals that were experimentally inoculated with *E. coli* O157:H7 American Type Culture Collection strain ATCC 43894 were the source of the manure analyzed in this study. These animals differentially shed the bacteria so that the feces collected were from both *E. coli* O157:H7 culture-positive and culture-negative animals. The manure piles were kept on cement floors in confined areas that were protected from direct precipitation but were exposed to climatic changes.

(i) Nonaerated ovine manure. Fecal material was collected from eight sheep that were inoculated with *E. coli* O157:H7 (36). The animals were housed on raised, grated flooring, and feces were collected beneath the pens for approximately 2 months (36). The animals were removed, and the pile of manure, which was 7 m long by 3 m wide by 0.6 m deep, was not disturbed; this manure is referred to below as nonaerated ovine manure. Every 30 days, 24 10-g samples were collected from the top, middle, and bottom layers of the manure pile and cultured to determine the presence of *E. coli* O157:H7, as described below. The genomic DNA profiles of the *E. coli* O157:H7 isolates cultured from the ovine manure pile were compared by pulsed-field gel electrophoresis (PFGE), as described below.

(ii) Aerated ovine manure. Feces from 23 sheep that were experimentally inoculated with *E. coli* O157:H7 (32) were collected daily for approximately 2 months and were divided into 27 small piles, each of which had a volume of approximately 50 cm^3. Ten-gram samples from the top, middle, and bottom layers of each pile were removed every 30 days and cultured to determine the presence of *E. coli* O157:H7 (see below). Prior to sampling, the manure in each pile was aerated by mixing. The manure from these piles is referred to below as aerated ovine manure.

(iii) Aerated bovine manure. Feces were collected from eight cattle that were inoculated with *E. coli* O157:H7 (7). Every day for approximately 2 months, manure was shoveled into a single pile away from the animals. Because the cattle were on wood chip bedding, small amounts (<5%) of this material were introduced into the fecal material. The manure was aerated by mixing it and was divided into 10 smaller piles, each of which had a volume of approximately 100 cm^3. Ten-gram samples collected from the middle of each of the 10 piles were cultured to determine the presence of *E. coli* O157:H7, as described below.

Culture. Each manure pile was analyzed to determine the presence of *E. coli* O157:H7 by previously described nonenrichment and selective-enrichment culture methods (36). Briefly, 10-g manure samples were transported to the laboratory in ice-cold Trypticase soy broth (BBL/Becton Dickinson) supplemented with cefixime (50 μg/liter; Lederle Laboratories, Pearle River, N.Y.), potassium tellurite (2.5 mg/liter; Sigma Chemical Co., St. Louis, Mo.), and vancomycin (40 mg/liter; Sigma). In the laboratory, appropriate serial dilutions of each sample, ranging from undiluted sample to a 10^{-10} dilution, were prepared with sterile saline (0.15 M NaCl) both before and after overnight aerated incubation at 37°C. The dilutions prepared before incubation were spread plated onto sorbitol MacConkey agar containing 4-methylumbelliferyl-β-D-glucuronide (100 mg/liter; Biosynth Ag Biochemica and Synthetica, Skokie, Ill.) (SMAC-MUG) (nonenrichment cultures). The dilutions prepared after overnight incubation were spread plated onto SMAC-MUG supplemented with cefixime (50 μg/liter) and potassium tellurite (2.5 mg/liter) (selective-enrichment cultures). With both methods colonies that did not ferment sorbitol and did not utilize 4-methylumbelliferyl-β-D-glucuronide were confirmed to be *E. coli* O157 serologically.

PFGE. Genomic DNA from *E. coli* O157:H7 isolates obtained after 1 and 12 months from the nonaerated ovine manure pile were prepared as previously described (5). At least five colonies were analyzed when the isolates were recovered by the nonenrichment culture technique. However, only two colonies were tested when the isolates were recovered by the selective-enrichment technique since they were probably clonal progeny. Each agarose-embedded DNA was digested with 10 U of *Xba*I (Gibco BRL, Grand Island, N.Y.) per plug at 37°C overnight. PFGE was performed with a CHEF-DR II unit (Bio-Rad Laboratories, Hercules, Calif.) by using 1% PFGE grade agarose–Tris borate buffer gels (Boehringer Mannheim, Indianapolis, Ind.) (5). The DNA was electrophoresed for 20 h at a constant voltage (200 V, 6 V/cm) with pulse times of 5 to 50 s and an electric field angle of 120° at a temperature of 15°C before being stained with ethidium bromide. The resulting patterns were analyzed with the ProRFLP program (DNA Proscan, Inc., Nashville, Tenn.), and the numbers and sizes of the DNA fragments were used as criteria for identifying distinct patterns.

Laboratory analysis of *E. coli* O157:H7 survival in farm effluents. (i) Sample collection. The common farm effluents analyzed included bovine and ovine feces and untreated and treated bovine slurries. These materials were collected from the dairy and sheep farms at the University of Idaho, Moscow, and Washington State University, Pullman. All materials were inoculated with *E. coli* O157:H7 within 1 h of collection. A 10-ml or 10-g sample of each material was tested for the presence of *E. coli* O157:H7 prior to inoculation by the nonenrichment and selective-enrichment methods described above.

(ii) Inoculum and incubation conditions. *E. coli* O157:H7 strains ATCC 43894 ($Stx1^+$ $Stx2^+$) and ATCC 43888 ($Stx1^-$ $Stx2^-$) were obtained from the American Type Culture Collection, Rockville, Md. The *E. coli* O157:H7 strains were grown in separate flasks containing 30 ml of Luria-Bertani broth (39) for 18 h at 37°C without agitation to an absorbance at 600 nm of 1.0 to 1.3 (~10^8 CFU/ml). Viable cell counts were determined in triplicate by serial dilution and spread plate culturing on Luria-Bertani agar. Cells were harvested by centrifugation, washed twice, resuspended in 2 ml of sterile saline (0.15 M NaCl), and used to inoculate the farm effluents. After inoculation, samples of each effluent aliquot were periodically collected and cultured to determine the presence of *E. coli* O157:H7 (see Fig. 2 through 4). The initial (zero-time postinoculation) *E. coli* O157:H7 concentration was determined for each fecal or slurry aliquot prior to incubation at various temperatures. All effluent samples were kept moist throughout the study by adding sterile saline when required.

(iii) Bovine farm effluents. Three hundred grams of feces, 300 ml of untreated slurry, and 300 ml of treated slurry were inoculated with *E. coli* O157:H7 strain ATCC 43894. After inoculation, each effluent material was mixed and divided into 30-g or 30-ml aliquots, and the aliquots were incubated at −20, 4, 23, 37, 45, and 70°C. The feces were incubated without aeration at all temperatures. The slurries were incubated without aeration (statically) at −20 and 70°C. At all other temperatures, duplicate samples of the slurries were incubated; one sample was aerated (by stirring), and the other sample was incubated statically. Samples were cultured to determine the presence of *E. coli* O157:H7 periodically for 28 days. Based on the survival of *E. coli* O157:H7 in the bovine effluents, fresh feces and fresh untreated slurry were inoculated with bacteria, incubated at −20, 4, 10, and 23°C without aeration, and monitored for 100 days. Duplicate 300-g or 30-ml aliquots of bovine feces and untreated slurry were inoculated with either ATCC 43894 or ATCC 43888. After inoculation, the effluent materials were mixed thoroughly and divided into 30-g or 30-ml portions for incubation at the different temperatures. Samples were cultured to determine the presence of *E. coli* O157:H7 periodically for 100 days.

(iv) Ovine feces. Similarly, 200 g of ovine feces was inoculated with ATCC 43894. After inoculation, the feces were mixed thoroughly, and 60-g aliquots were incubated at 4, 10, and 23°C without aeration. Samples were cultured to determine the presence of *E. coli* O157:H7 periodically for 100 days.

DNA probes and colony blot DNA hybridization. Colony blots were prepared from both bovine feces and untreated slurry culture plates at all sampling times during the 100-day study and were probed for the presence of the toxin genes stx_1 and stx_2. The gene probes for stx_1 and stx_2 were derived from the 656-bp *Pst*I-*Hin*dIII fragment of pSC25 (25) and the 842-bp *Sma*I-*Pst*I fragment of pMJ331 (62), respectively. Each probe was labeled with [α-^{32}P]dCTP by using the Radprime DNA labeling system (Gibco BRL). Colony blots were prepared with Nytran membranes (pore size, 0.45 μm; Schleicher and Schuell, Keene, N.H.) and were hybridized with the labeled probes by using standard protocols (39).

RESULTS

Survival of *E. coli* O157:H7 in manure exposed to the environment. The average concentrations of the cultured background flora in the manure ranged from 10^5 to 10^8 CFU/g at the start of the study. After 12 months, the background flora concentrations declined to 10^1 to 10^2 CFU/g in the nonaerated ovine manure pile. In the aerated ovine manure and bovine manure piles the detectable background flora concentrations remained 10^5 to 10^6 CFU/g for the duration of the studies.

(i) Nonaerated ovine manure pile. Except for the samples taken at month 4 (November), *E. coli* O157:H7 was consistently isolated from the nonaerated ovine manure pile for 12 months (Table 1). The bacteria were recovered by the selective-enrichment technique from the moist middle and bottom layers of the manure pile but not from the dry fecal material at the top of the pile. *E. coli* O157:H7 was isolated by the nonenrichment culture technique only at three samplings times and only from the middle layer of the pile (Table 1). The

TABLE 1. Isolation of *E. coli* O157:H7 from manure exposed to the environment

Manure pile	Month	Culture status
Nonaerated ovine[a]	August	SE+[d]
	September	SE+
	October	SE+
	November	−[e]
	December	SE+
	January	NE+ (2.2×10^6 CFU/g)[f]
	February	SE+
	March	SE+
	April	SE+
	May	SE+
	June	NE+ (4.5×10^3 CFU/g)
	July	NE+ (3.7×10^4 CFU/g)
	August	SE+
	September	−
	October	−
Aerated ovine[b]	October	SE+
	November	−
	December	SE+
	January	SE+
	February	−
	March	−
Aerated bovine[c]	March	SE+
	April	SE+
	May	−
	June	−

[a] The manure was in one pile. Isolates were recovered from the middle and bottom layers and were collected from six sites.
[b] A total of 27 piles were analyzed, and isolates were recovered from the middle and bottom layers of 8 piles.
[c] Ten piles were analyzed, and *E. coli* O157:H7 was isolated from the middle layers of all piles.
[d] SE+, selective-enrichment culture positive only. The *E. coli* O157:H7 concentrations were $\leq 10^2$ CFU/g.
[e] −, culture negative for *E. coli* O157:H7.
[f] NE+, nonenrichment culture positive. The *E. coli* O157:H7 concentrations (values in parentheses) are averages of the data obtained at two or three sites.

highest concentration of *E. coli* O157:H7 detected in the manure was 2.2×10^6 CFU/g. As shown in Fig. 1, the DNA fingerprints (PFGE genomic DNA profiles) of *E. coli* O157:H7 isolated at month 1 and month 12 were identical (Fig. 1, lanes 2 and 3). The DNA fingerprints were identical to the DNA fingerprint of strain ATCC 43894, the strain that was used to inoculate the sheep whose feces made up the manure pile (Fig. 1, lane 1). An *E. coli* O157:H7 isolate with a very similar DNA fingerprint was isolated from the manure pile at month 12 of the experiment (Fig. 1, lane 4). This variant of the original *E. coli* O157:H7 strain lacked a DNA fragment that migrated at the 193-kb position.

At months 13 and 14, cultures from the manure were negative for *E. coli* O157:H7. The manure pile was not cultured for the following 6 months but was left undisturbed. Twenty-one months after the start of the experiment, the manure pile was tested and was culture positive, as determined by the selective-enrichment culture method. By this time, however, the concentration of *E. coli* O157:H7 had declined significantly so that the bacteria were recovered from only 1 of 24 samples (data not shown).

(ii) Aerated ovine manure piles. *E. coli* O157:H7 was isolated intermittently by the selective-enrichment culture method from the moist middle and bottom layers of 8 of the 27 aerated ovine manure piles tested (Table 1). *E. coli* O157:H7 was recovered from these eight culture-positive manure piles for 4 months. The remaining 19 manure piles were consistently culture negative for *E. coli* O157:H7 from the beginning of the experiment.

(iii) Aerated bovine manure piles. *E. coli* O157:H7 was isolated by the selective-enrichment culture method from the moist middle layers of all 10 aerated bovine manure piles for 47 days (Table 1).

Survival of *E. coli* O157:H7 in farm effluents under laboratory conditions. All farm effluents used in this study were *E. coli* O157:H7 negative prior to inoculation, as determined by culturing. The presence of viable but noncultivable *E. coli* O157:H7 in the effluents was not determined. To compare the survivability of *E. coli* O157:H7 with the survivability of the competing background flora, effluents were inoculated with concentrations of *E. coli* O157:H7 similar to the concentrations of the indigenous flora. First, inoculated bovine farm effluents were incubated under eight different culture conditions, and the survival of *E. coli* O157:H7 was monitored for 28 days (Fig. 2). Based on the survival of *E. coli* O157:H7 in these studies, fresh bovine feces, ovine feces, and untreated bovine slurry were inoculated, incubated under four culture conditions, and monitored for 100 days (Fig. 3 and 4). In the laboratory, waste materials were kept moist with saline. Although the surfaces of most fecal piles and slurry lagoons tend to dry out over time, it was our observation that the interiors of our preparations always remained moist. In addition, we recovered *E. coli* O157:H7 from manure piles only from the moist interior layers and not from dried surface samples. Throughout these studies, the pH of each reaction mixture was measured, and the values ranged from pH 7.0 to 9.0. There was no correlation between incubation temperature or the survival of *E. coli* O157:H7 and pH.

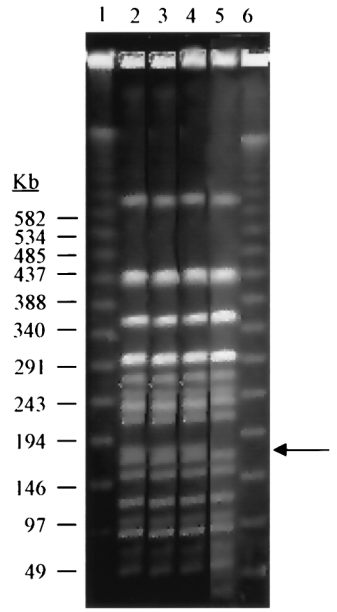

FIG. 1. PFGE profiles of *E. coli* O157:H7 isolated from the nonaerated ovine manure pile. Lane 2, ATCC 43894 (inoculated into the sheep whose feces contributed to the pile); lane 3, *E. coli* O157:H7 isolated from the manure during month 1 (and at all sampling times); lane 4, *E. coli* O157:H7 isolated from the manure during month 12 (and at all sampling times); lane 5, variant *E. coli* O157:H7 isolated from the manure during month 12 (the arrow indicates a missing band); lanes 1 and 6, bacteriophage lambda DNA ladder standard used for PFGE applications (Bio-Rad). The numbers on the left indicate molecular sizes.

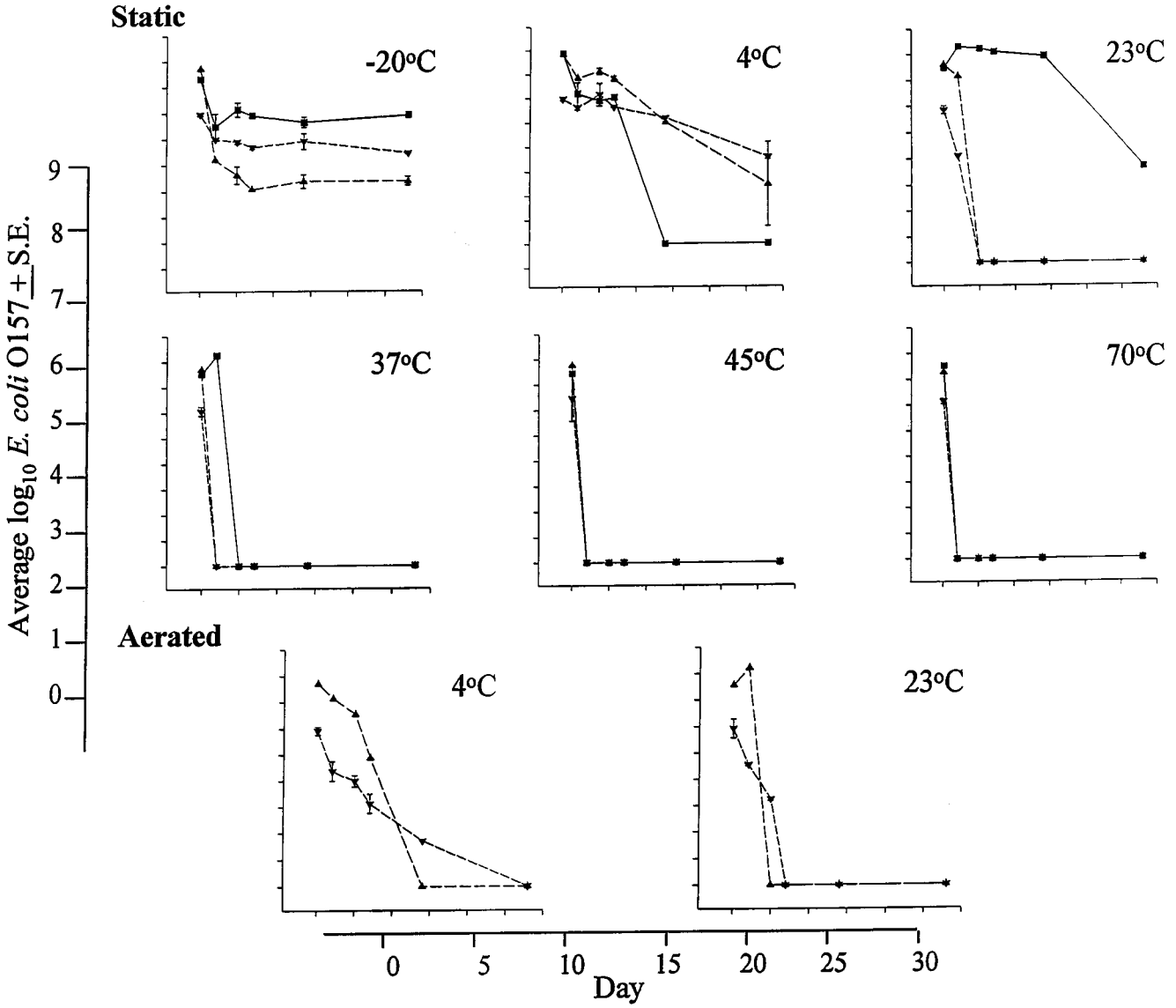

FIG. 2. Survival of *E. coli* O157:H7 in bovine farm effluents. Bovine feces (■), untreated slurry (▲), and treated slurry (▼) were inoculated and monitored for 28 days in the laboratory. Aliquots of the inoculated farm effluents were incubated at different temperatures with aeration (aerated) or without aeration (static). The average concentrations of *E. coli* O157:H7 were determined by the nonenrichment culture method and are shown as $\log_{10}$ CFU per gram and $\log_{10}$ CFU per milliliter for feces and slurries, respectively.

(i) Bovine feces monitored for 28 days. Fecal material was not aerated, and 300 g was inoculated with 6.6×10^9 CFU of *E. coli* O157:H7 strain ATCC 43894. Immediately after inoculation, the average preincubation concentration of *E. coli* O157:H7 was 3.6×10^7 CFU/g. After incubation, the bacteria survived for the longest times at low temperatures (−20, 4, and 23°C) (Fig. 2). After incubation at higher temperatures, bacteria were detected at one (37°C) or none (45 or 70°C) of the sampling times (Fig. 2). The concentration of *E. coli* O157:H7 in frozen (−20°C) fecal material or feces incubated at 4°C decreased by 2 logs 48 h postinoculation but remained constant thereafter (Fig. 2). In contrast, after 24 h of incubation at 23 or 37°C, the concentration of *E. coli* O157:H7 increased by about 1 log. Continued incubation at 37°C resulted in a rapid decline in the number of viable bacteria, while continued incubation at 23°C did not result in a decrease in the *E. coli* O157:H7 concentration below the inoculation concentration for at least 14 days (Fig. 2).

(ii) Untreated bovine slurry monitored for 28 days. Three hundred milliliters of untreated slurry was inoculated with 6.1×10^9 CFU of *E. coli* O157:H7 strain ATCC 43894. The average preincubation concentration of *E. coli* O157:H7 was 4.3×10^7 CFU/ml. After incubation, *E. coli* O157:H7 was isolated from the −20 and 4°C nonaerated cultures at all sampling times and from the 4°C aerated culture until day 7 postinoculation (Fig. 2). At 23°C, both the aerated and nonaerated cultures yielded *E. coli* O157:H7 only on day 2 postinoculation (Fig. 2). At the higher temperatures (37, 45, and 70°C), the slurry was *E. coli* O157:H7 negative. In slurry incubated at −20°C, a 2-log decrease in the *E. coli* O157:H7 concentration occurred by day 2 postinoculation, compared to the 1-log decrease observed in samples incubated at 4°C (Fig. 2).

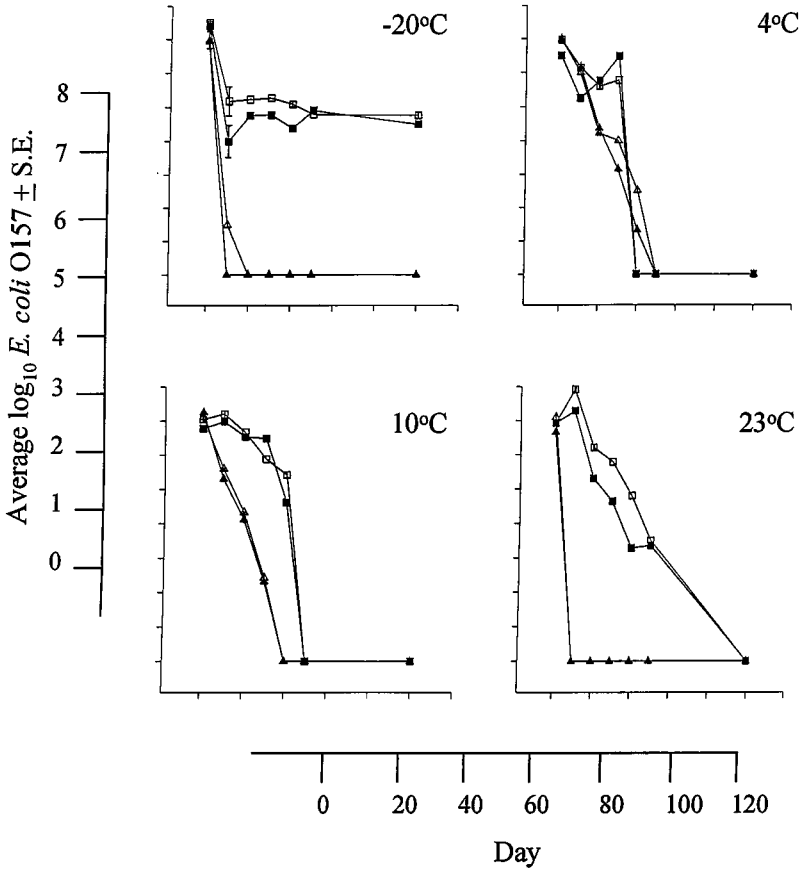

FIG. 3. Comparison of toxin-positive and toxin-negative *E. coli* O157:H7 survival in inoculated manure. Bovine feces were inoculated with toxin-negative *E. coli* O157:H7 strain ATCC 43888 (■) or toxin-positive *E. coli* O157 strain ATCC 43894 (□), or bovine untreated slurry was inoculated with toxin-negative *E. coli* O157 strain ATCC 43888 (▲) or toxin-positive *E. coli* O157 strain ATCC 43894 (△). Aliquots of the inoculated farm effluents were incubated at various temperatures without aeration in the laboratory and monitored for 100 days. The average concentrations of *E. coli* O157:H7 were determined by the nonenrichment culture method and are shown as $\log_{10}$ CFU per gram and $\log_{10}$ CFU per milliliter for feces and slurry, respectively.

(iii) Treated bovine slurry monitored for 28 days. Three hundred milliliters of treated slurry was inoculated with 6.3×10^9 CFU of ATCC 43894. The average preincubation concentration of *E. coli* O157:H7 in aliquots of this effluent was 9.7×10^5 CFU/ml. After 2 days of static incubation at −20 or 4°C, the concentration of *E. coli* O157:H7 had declined by about 1 order of magnitude, but then it remained constant through 28 days postinoculation (Fig. 2). With aeration at 4°C, however,

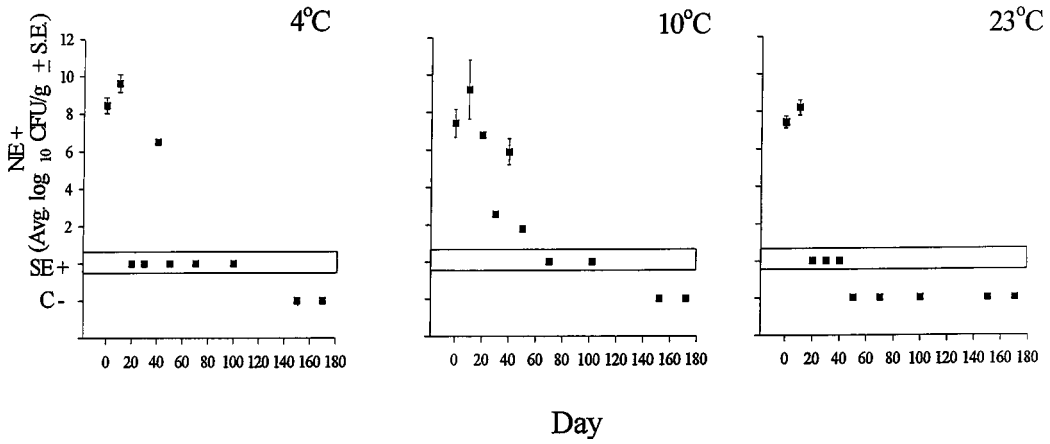

FIG. 4. Survival of *E. coli* O157:H7 in inoculated ovine feces monitored for 170 days in the laboratory. Aliquots of the inoculated feces were incubated at three temperatures without aeration. The average concentrations of *E. coli* O157:H7 were determined by the nonenrichment culture method (NE+). Fecal cultures that were positive only as determined by the selective-enrichment method (SE+) are indicated in the boxes. C−, culture negative.

the concentration of the bacteria declined after 14 days postinoculation (Fig. 2). At all other temperatures the organism was isolated either for a short period of time (23°C) or not at all (37, 45, and 70°C) (Fig. 2).

(iv) Bovine feces inoculated with toxin-positive or toxin-negative strains of *E. coli* O157:H7 and monitored for 100 days. One 300-g aliquot of fecal material was inoculated with 5.3×10^9 CFU of ATCC 43888, and another 300-g aliquot of fecal material was inoculated with 5.1×10^9 CFU of ATCC 43894. The average preincubation *E. coli* O157:H7 concentration for eight 30-g aliquots was 1.41×10^7 CFU/g. After incubation, almost identical patterns of survival were observed for the two strains of *E. coli* O157:H7 (Fig. 3). No significant difference was found in the patterns of recovery of the two strains of *E. coli* O157:H7 in feces incubated at 10 or 4°C. During the first 40 days of incubation, toxin-positive strain ATCC 43894 survived better than toxin-negative strain ATCC 43888 in manure incubated at −20 or 23°C. Similar concentrations of the two strains were isolated from feces frozen at −20°C for at least 100 days postinoculation. Likewise, both strains were recovered for shorter lengths of time following incubation at higher temperatures.

(v) Untreated bovine slurry inoculated with toxin-positive and toxin-negative strains of *E. coli* O157:H7 and monitored for 100 days. The untreated bovine slurry used in the 100-day study was more dilute than the slurry used in the 28-day study (see above). One 300-ml aliquot of untreated slurry was inoculated with 5.2×10^9 CFU of ATCC 43888, and another 300-ml aliquot was inoculated with 8.4×10^9 CFU of ATCC 43894. The average preincubation *E. coli* O157:H7 concentration for eight 30-g aliquots was 6.1×10^6 CFU/g. Unlike recovery of the bacteria from frozen feces, the organism was not isolated from the slurry after incubation at −20 or 23°C but was recovered after 40 and 30 days of incubation at 4 and 10°C, respectively (Fig. 3). No significant difference was found in the patterns of recovery of the two strains of *E. coli* O157:H7 from the slurry except after 7 days of incubation at −20°C, when the toxin-positive strain had a slight survival advantage.

DNA hybridization confirmed that the toxin genes were present in strain ATCC 43894. The stx_1 and stx_2 gene probes hybridized only with the *E. coli* O157:H7 isolates recovered from the effluents inoculated with strain ATCC 43894 (data not shown). The probes did not hybridize with any of the background non-O157 isolates or with the *E. coli* O157:H7 isolates that originated from ATCC 43888.

(vi) Ovine feces: 100-day study. Two hundred grams of ovine feces was inoculated with 5×10^9 CFU of ATCC 43894. The average preincubation *E. coli* O157:H7 concentration for three 60-g aliquots was 1×10^8 CFU/g. Like the survival of this bacterium in bovine feces, the concentration of *E. coli* O157:H7 declined with incubation. As expected, the selective-enrichment culture method was the most sensitive method for detecting the bacteria. The organism was detected by the nonenrichment culture method for the longest time (50 days) after incubation at 10°C and was detected by this culture technique after incubation at 4 and 23°C for 40 and 10 days, respectively (Fig. 4). However, the bacteria were detected by the selective-enrichment technique at day 100 postinoculation after incubation at 4 and 10°C and at day 40 postinoculation after incubation at 23°C (Fig. 4).

Indigenous bacteria cultured from the bovine farm effluents and ovine feces. Prior to *E. coli* O157:H7 inoculation, the concentration of bacteria cultured by spread plating on SMAC-MUG was determined for each of the effluent samples used. In the samples used for the 28-day study the concentrations were as follows: 2.04×10^7 CFU/g of bovine feces, 1.02×10^6 CFU/ml of untreated slurry, and 2.35×10^6 CFU/ml of treated slurry. Similarly, in the samples used for the 100-day study, the concentrations were as follows: 4.35×10^8 CFU/g of bovine feces, 2.36×10^6 CFU/ml of untreated bovine slurry, and 1.15×10^8 CFU/g of ovine feces.

Following inoculation with concentrations of *E. coli* O157:H7 similar to the concentrations of the background flora cultured, the pathogen was the predominant bacterium recovered in all cultures. However, as the number of *E. coli* O157:H7 cells declined, more background bacteria were isolated (data not shown). The total number of bacteria declined with time. In general, after incubation at low temperatures (−20, 4, 10, and 23°C) *E. coli* O157:H7 was the predominant bacterium, while after incubation at 37 or 45°C the background flora dominated the cultures. No bacteria were isolated after incubation at 70°C.

DISCUSSION

The most significant finding of this work is that *E. coli* O157:H7 survived for more than 1 year in a nonaerated ovine manure pile that was exposed to environmental conditions. In similar aerated ovine manure and bovine manure piles, the organism survived for 4 months and 47 days, respectively. The finding that *E. coli* O157:H7 can survive in the environment for a long time has implications for understanding the ecology of this human pathogen in its ruminant reservoirs and in the farm environment.

The potential risk to human and animal health of irrigating land with slurry has often been addressed (15, 23, 40, 60). In fact, direct or indirect contact with animals and their by-products, such as feces and slurry, has been implicated in several human cases of *E. coli* O157:H7 infection. Renwick et al. were the first workers to report direct transmission of *E. coli* O157:H7 from calves to a child via the fecal-oral route (45). Recently, two cases of *E. coli* O157:H7 infection in Scotland were linked to direct contact with infected sheep feces (3). Manure-contaminated vegetables have caused a number of outbreaks, including an outbreak in Japan that affected about 10,000 people (58). In addition, Mechie et al. recorded a possible correlation between a high incidence of *E. coli* O157:H7 in heifers and previous exposure of the animals to a silage field irrigated with slurry (40).

In the past, animal waste and bedding were composted for several days, and the compost reached temperatures of 70°C or more before being used as fertilizer (26, 64). Composting and drying of manure is known to reduce the number of viable pathogens (26, 42, 64). While composting is ideal, it is no longer a practical approach for processing cattle manure. Advancements in mechanized farming have led to large numbers of animals per farm, and quick and easy methods for disposal of wastes have been devised. Most large farms wash animal feces, urine, and spilt feed into a slurry mixture (64). The slurry is held in settling tanks away from the animal housing and undergoes anaerobic degradation (untreated slurry) for more than 1 month before disposal (26, 64). In addition, some farms reduce the bulk of untreated slurry by using a mechanical aeration technique that separates the solid and liquid portions of the slurry. Appropriately treated liquids are released into the environment, while the solids (treated slurry), which occupy less space, are degraded by anaerobiosis before being used as fertilizer (26, 64). Farm effluents should be contained in holding tanks with proper aeration for appropriate lengths of time (1 to 3 months or as required) before being used as fertilizers (50). Improperly incubated and/or stored slurry can serve as a vehicle for environmental spread and propagation of

pathogens that may include *E. coli* O157:H7 (15, 50, 53, 56, 61).

In an effort to determine the risk posed by *E. coli* O157:H7-contaminated manure, we monitored the survival of this pathogen in ovine and bovine manure piles obtained from experimentally infected animals. These manure piles were exposed to climatic conditions and were either left undisturbed or aerated by manual mixing. Twenty-one months after the start of the experiment, we were able to culture *E. coli* O157:H7 from a nonaerated ovine manure pile. Because the *E. coli* O157:H7 recovered from this manure had the same DNA fingerprint as the *E. coli* O157:H7 used to inoculate the sheep that created the manure, it is highly unlikely that a new contaminant was introduced into the manure. In addition, a variant of the *E. coli* O157:H7 strain was cultured from this manure pile, and this variant differed from the original strain by a single PFGE band, which may be indicative of a mutation or a loss of plasmid DNA (27, 28, 41). This slightly different strain was not the predominant organism, and the alteration may have occurred in the sheep GIT or in the manure pile. The concentrations of *E. coli* O157:H7 recovered from this manure ranged from $<10^2$ CFU/g (detectable only by the selective-enrichment culture method) to 10^6 CFU/g. The months during which the highest concentration of *E. coli* O157 were found coincided with months when the daily temperature increased. For example, the samples obtained in June and July contained 10^4 and 10^5 CFU of *E. coli* O157:H7/g, respectively. Also, in January there were several days of unseasonably high temperatures (data not shown) before a concentration of 10^6 CFU of *E. coli* O157:H7/g was found in the samples. The different concentrations may reflect differences in the distribution of the bacteria in the pile. However, the concentration found in the January samples, 10^6 CFU of *E. coli* O157:H7/g, was greater than the *E. coli* O157 concentration in the feces of the sheep that created the manure pile. The average concentration of *E. coli* O157:H7 in feces taken from animals by aseptic rectal palpation was about 10^3 CFU/g, and the highest concentration was 10^5 CFU/g (36).

The differences between the lengths of time that *E. coli* O157:H7 survived in aerated ovine manure (4 months) and aerated cattle manure (47 days) and the lengths of time that this organism survived in nonaerated manure may be a direct result of drying. *E. coli* O157:H7 was never recovered from the dry top layer of any manure pile. Relatively large amounts of manure were dried due to periodic mixing and the larger surface area of the smaller aerated manure piles compared to the large nonaerated ovine manure pile. Controlled experiments should be conducted to determine if dehydration is the cause of the differences in survival times. The small amounts of wood chip bedding in the bovine manure may also have contributed to the shorter *E. coli* O157:H7 survival times. Interestingly, *E. coli* O157:H7 survived longer in manure (ovine or bovine) in the environment than in the GIT of the animals. The average lengths of time that a sheep and a cow shed the bacteria in feces were 22 and 20 days, respectively (7, 36). Potential complex differences in microbial ecology between digesta in the ruminant GIT and manure in the environment may have affected survival of *E. coli* O157:H7 in these settings.

It is well-established that infection of cattle and sheep with *E. coli* O157:H7 follows a seasonal pattern, with the highest incidence of culture-positive animals occurring in the warmer months (22, 34). The finding that *E. coli* O157:H7 is able to survive in the environment year-round suggests that it may be reintroduced into cattle and sheep from a contaminated farm environment. Previously, Rahn et al. isolated the same phage type of *E. coli* O157:H7 on the same farm two times 12 months apart (44). Faith et al. showed that *E. coli* O157:H7 isolates from a single farm often had identical fingerprints (restriction endonuclease digestion profiles) (16). However, over an 8-month period animals in one herd usually contained isolates with more than one fingerprint, suggesting that new strains are introduced on farms over time. These new strains may be acquired from new animals, fresh feed, water, birds, flies, or deer (24, 30, 37, 44).

Although we did not determine if the contaminated manure was infectious for ruminants, it is very likely that it was. It has been shown that a single dose of 10^2 CFU of *E. coli* O157:H7 is sufficient to infect cattle (24). Although the lower limit of an infectious dose for sheep has not been determined, lambs can be infected by a single oral dose of 10^5 CFU of *E. coli* O157:H7 (33). Ingestion of very small amounts (0.0001 or 0.1 g) of manure containing 10^6 CFU/g would be required to reach these levels of inoculation. In addition, the estimated infectious dose for humans is as low as 10 bacteria (23, 53).

Laboratory experiments performed to mimic farm effluents in the environment confirmed that *E. coli* O157:H7 survived best in effluents with a high solid content incubated without aeration at temperatures below 23°C (manure incubated at −20, 4, or 10°C). *E. coli* O157:H7 was not efficiently recovered from effluents incubated at higher temperatures (37, 45, or 70°C). These findings confirm the earlier finding of Wang et al. (60). We also observed a 1- to 2-log reduction in the *E. coli* O157:H7 concentration immediately after inoculation and prior to incubation in the treated slurry. This suggests that treated slurry has an inhibitory effect that should be studied. Although we did not measure the solid content, other studies have clearly demonstrated the influence of high percentages of solids in slurries on the extended growth of bacteria in slurries (26, 64). The results of studies performed with inoculated ovine feces were similar to the results of studies performed with bovine effluents and showed that the pathogen survived the longest (100 days) during incubation at 10 or 4°C. However, unlike the recovery of *E. coli* O157:H7 from bovine effluents, the selective-enrichment culture method was required to recover the bacteria from the ovine feces. This finding that *E. coli* O157:H7 survives poorly in ovine feces is in complete contrast to our observations obtained with ovine manure piles kept in the environment.

We also investigated the role of the toxin genes stx_1 and stx_2 in *E. coli* O157:H7 survival in bovine manure and untreated slurry. It is well-documented that Shiga toxin is cytotoxic to some eukaryotic cells (21, 31, 38), and it has been hypothesized that this cytotoxicity might help *E. coli* O157:H7 survive in the environment. However, a very slight influence was observed under the laboratory conditions tested, as identical or very similar survival patterns were observed with toxin-positive and toxin-negative strains of *E. coli* O157:H7. Since the strains used in this analysis were not isogenic, factors other than toxin may have contributed to the minor survival differences.

Interestingly, under most conditions, *E. coli* O157:H7 survived less well in experimentally inoculated farm effluents (manure, untreated slurry, or treated slurry) incubated under laboratory conditions than in naturally inoculated manure in the environment. There may be for a variety of reasons for this. The naturally inoculated manure contained bacteria that had passed through the ruminant GIT, and this may have selected for bacteria that were well-suited to survive. Also, the physical dimensions of the manure piles may have provided a niche(s) that could not be reproduced in the laboratory. These findings emphasize the difficulties in extrapolating laboratory data to on-farm conditions. Finally, our observations confirm that appropriate farm waste management plays a critical role in preventing the persistence of *E. coli* O157:H7 on the farm.

ACKNOWLEDGMENTS

This work was supported in part by the Idaho Agriculture Experiment Station, by U.S. Department of Agriculture NRICGP grants 92-04350 and 95-37201-1979, by Public Health Service grant AI33981 from the National Institutes of Health, and by a grant from the United Dairymen of Idaho and the Idaho Beef Council.

We acknowledge the technical assistance provided by U. M. Nance, D. K. Gaskin, S. Haenny, P. R. Austin, and K. Cloud. We thank D. D. Hancock, Washington State University, Pullman, for providing cefixime and D. Rice, Washington State University, Pullman, for PFGE analyses.

REFERENCES

1. **Acheson, D. W. K., A. Donohue-Rolfe, and G. T. Keusch.** 1991. The family of Shiga and Shiga-like toxins, p. 415–433. *In* J. E. Alouf and J. H. Freer (ed.), Sourcebook of bacterial toxins. Academic Press Inc., London, United Kingdom.
2. **Ajariyakhajorn, C., S. M. Goyal, R. A. Robinson, L. J. Johnston, and C. A. Clanton.** 1997. The survival of *Salmonella anatum*, pseudorabies virus and porcine reproductive and respiratory syndrome virus in swine slurry. New Microbiol. **20:**365–369.
3. **Allison, L., P. Carter, and F. Thomson-Carter.** 1997. Pinpointing human infection of *E. coli* O157 from animals, abstr. no. V84/I, p. 10. *In* VTEC'97, 3rd International Symposium and Workshop on Shiga Toxin (Verocytotoxin)-Producing *Escherichia coli* Infections.
4. **Banatvala, N., A. R. Magnano, M. L. Cartter, T. J. Barrett, W. F. Bibb, L. L. Vasile, P. Mshar, M. A. Lambert-Fair, J. H. Green, N. H. Bean, and R. V. Tauxe.** 1996. Meat grinders and molecular epidemiology: two supermarket outbreaks of *Escherichia coli* O157:H7 infection. J. Infect. Dis. **173:**480–483.
5. **Barrett, T. J., H. Lior, J. H. Green, R. Khakhria, J. G. Wells, B. P. Bell, K. D. Greene, J. Lewis, and P. M. Griffin.** 1994. Laboratory investigation of a multistate food-borne outbreak of *Escherichia coli* O157:H7 by using pulsed-field gel electrophoresis and phage typing. J. Clin. Microbiol. **32:**3013–3017.
6. **Besser, R. E., S. M. Lett, J. T. Weber, M. P. Doyle, T. J. Barrett, J. G. Wells, and P. M. Griffin.** 1993. An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh-pressed apple cider. JAMA **269:**2217–2220.
7. **Bohach, C. H., P. R. Austin, S. Haenny, I. T. Kudva, and C. W. Hunt.** The effect of dietary protein, fiber and digesta passage rate on the duration of *Escherichia coli* O157:H7 shed by cattle. *In* Proceedings of the 1998 Meeting of the American Association of Bovine Practitioners, in press.
8. **Boyce, T. G., D. L. Swerdlow, and P. M. Griffin.** 1995. Current concepts—*Escherichia coli* O157:H7 and the hemolytic-uremic syndrome. N. Engl. J. Med. **333:**364–368.
9. **Centers for Disease Control and Prevention.** 1995. *Escherichia coli* O157:H7 outbreak linked to commercially distributed dry-cured salami—Washington and California, 1994. Morbid. Mortal. Weekly Rep. **44:**157–160.
10. **Centers for Disease Control and Prevention.** 1996. Outbreak of *Escherichia coli* O157:H7 infections associated with drinking unpasteurized commercial apple juice—British Columbia, California, Colorado, and Washington, October 1996. Morbid. Mortal. Weekly Rep. **45:**975.
11. **Centers for Disease Control and Prevention.** 1997. Outbreaks of *Escherichia coli* O157:H7 infection and cryptosporidiosis associated with drinking unpasteurized apple cider—Connecticut and New York, October 1996. Morbid. Mortal. Weekly Rep. **46:**5–8.
12. **Chapman, P. A., C. A. Siddons, D. J. Wright, P. Norman, J. Fox, and E. Crick.** 1993. Cattle as a possible source of verocytotoxin-producing *Escherichia coli* O157 infections in man. Epidemiol. Infect. **111:**439–447.
13. **Cieslak, P. R., T. J. Barrett, P. M. Griffin, K. F. Gensheimer, G. Beckett, J. Buffington, and M. G. Smith.** 1993. *Escherichia coli* O157:H7 infection from a manured garden. Lancet **342:**8867.
14. **Deng, M. Y., and D. O. Cliver.** 1995. Persistence of inoculated hepatitis A virus in mixed human and animal wastes. Appl. Environ. Microbiol. **61:**87–91.
15. **Dorn, C. R.** 1993. Review of foodborne outbreak of *Escherichia coli* O157:H7 infection in the western United States. J. Am. Vet. Med. Assoc. **203:**1583–1587.
16. **Faith, N. G., J. A. Shere, R. Brosch, K. W. Arnold, S. E. Ansay, M. S. Lee, J. B. Luchansky, and C. W. Kaspar.** 1996. Prevalence and clonal nature of *Escherichia coli* O157:H7 on dairy farms in Wisconsin. Appl. Environ. Microbiol. **62:**1519–1525.
17. **Forshell, L. P., and I. Ekesbo.** 1993. Survival of salmonellas in composted and not composted solid animal manure. Zentralbl. Veterinaermed. **40:**654–658.
18. **Goodger, W. J., M. T. Collins, K. V. Nordlund, C. Eisele, J. Pelletier, C. B. Thomas, and D. C. Sockett.** 1996. Epidemiologic study of on-farm management practices associated with prevalence of *Mycobacterium paratuberculosis* infections in dairy cattle. J. Am. Vet. Med. Assoc. **208:**1877–1881.
19. **Griffin, P. M.** 1995. *Escherichia coli* O157:H7 and other enterohemorrhagic *Escherichia coli*, p. 739–761. *In* M. F. Blaser, P. D. Smith, J. I. Ravdin, H. B. Greenberg, and R. L. Guerrant (ed.), Infections of the gastrointestinal tract. Raven Press, Ltd., New York, N.Y.
20. **Griffin, P. M., and R. V. Tauxe.** 1991. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. Epidemiol. Rev. **13:**60–98.
21. **Gyles, C. L.** 1994. *Escherichia coli* in domestic animals and humans. CAB International, Oxon, United Kingdom.
22. **Hancock, D. D., T. E. Besser, M. L. Kinsel, P. I. Tarr, D. H. Rice, and M. G. Paros.** 1994. The prevalence of *Escherichia coli* O157:H7 in dairy and beef cattle in Washington state. J. Epidemiol. Infect. **113:**199–207.
23. **Hancock, D. D., T. E. Besser, and D. H. Rice.** 1993. Macro- and micro-epidemiology of *E. coli* O157:H7—prospects for pre-harvest control. *In* 97th Annual Meeting of the U.S. Animal Health Association.
24. **Hancock, D. D., T. E. Besser, and D. H. Rice.** 1997. The ecology of *Escherichia coli* O157:H7 in cattle and the impact of management practices. *In* VTEC'97, 3rd International Symposium and Workshop on Shiga Toxin (Verocytotoxin)-Producing *Escherichia coli* Infections.
25. **Hovde, C. J., S. B. Calderwood, J. J. Mekalanos, and R. J. Collier.** 1988. Evidence that glutamic acid 167 is an active-site residue of Shiga-like toxin I. Proc. Natl. Acad. Sci. USA **85:**2568–2572.
26. **Jones, P. W.** 1980. Animal health today—problems of large livestock units. Disease hazards associated with slurry disposal. Br. Vet. J. **136:**529–540.
27. **Karch, H., J. Heesemann, R. Laufs, A. D. O'Brien, C. O. Tacket, and M. M. Levine.** 1987. A plasmid of enterohemorrhagic *Escherichia coli* O157:H7 is required for expression of a new fimbrial antigen and for adhesion to epithelial cells. Infect. Immun. **55:**455–461.
28. **Karch, H., T. Meyer, H. Russman, and J. Heesemann.** 1992. Frequent loss of Shiga-like toxin genes in clinical isolates of *Escherichia coli* upon subcultivation. Infect. Immun. **60:**3464–3467.
29. **Karmali, M. A., M. Petric, C. Lim, P. C. Fleming, G. S. Arbus, and H. Lior.** 1985. The association between idiopathic hemolytic uremic syndrome and infection by verotoxin producing *Escherichia coli*. J. Infect. Dis. **151:**775–782.
30. **Keene, W. E., E. Sazie, J. Kok, D. H. Rice, D. D. Hancock, V. K. Balan, T. Zhao, and M. P. Doyle.** 1997. An outbreak of *Escherichia coli* O157:H7 infections traced to jerky made from deer meat. JAMA **277:**1229–1231.
31. **Konowalchuk, J., N. Dickie, S. Stavric, and J. I. Speirs.** 1978. Properties of an *Escherichia coli* cytotoxin. Infect. Immun. **20:**575–577.
32. **Kudva, I. T.** 1997. Analysis of *Escherichia coli* O157:H7 colonization of sheep, a new ruminant reservoir. Ph.D. dissertation. University of Idaho, Moscow.
33. **Kudva, I. T., P. G. Hatfield, and C. J. Hovde.** 1995. Effect of diet on the shedding of *Escherichia coli* O157:H7 in a sheep model. Appl. Environ. Microbiol. **61:**1363–1370.
34. **Kudva, I. T., P. G. Hatfield, and C. J. Hovde.** 1997. Characterization of *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* isolated from sheep. J. Clin. Microbiol. **35:**892–899.
35. **Kudva, I. T., P. G. Hatfield, and C. J. Hovde.** 1996. *Escherichia coli* O157:H7 in microbial flora of sheep. J. Clin. Microbiol. **34:**431–433.
36. **Kudva, I. T., C. W. Hunt, C. J. Williams, U. M. Nance, and C. J. Hovde.** 1997. Evaluation of dietary influences on *Escherichia coli* O157:H7 shedding by sheep. Appl. Environ. Microbiol. **63:**3878–3886.
37. **LeJeune, J., D. D. Hancock, and T. E. Besser.** 1997. *Escherichia coli* O157:H7 in cattle water troughs: a possible on-farm reservoir. *In* Fifth Annual Food Safety Farm to Table Conference of the Northwest Food Safety Consortium. Northwest Food Safety Consortium, Moscow, Idaho.
38. **Levine, M. M.** 1987. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J. Infect. Dis. **155:**377–389.
39. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. **Mechie, S. C., P. A. Chapman, and C. A. Siddons.** 1997. A fifteen month study of *Escherichia coli* O157:H7 in a dairy herd. Epidemiol. Infect. **118:**17–25.
41. **Paros, M., P. I. Tarr, H. Kim, T. E. Besser, and D. D. Hancock.** 1993. A comparison of human and bovine *Escherichia coli* O157:H7 isolates by toxin genotype, plasmid profile, and bacteriophage lambda-restriction fragment length polymorphism profile. J. Infect. Dis. **168:**1300–1303.
42. **Pell, A. N.** 1997. Manure and microbes: public and animal health problem? J. Dairy Sci. **80:**2673–2681.
43. **Pesaro, F., I. Sorg, and A. Metzler.** 1995. In situ inactivation of animal viruses and a coliphage in nonaerated liquid and semiliquid animal wastes. Appl. Environ. Microbiol. **61:**92–97.
44. **Rahn, K., S. A. Renwick, R. P. Johnson, J. B. Wilson, R. C. Clarke, D. Alves, S. McEwen, H. Lior, and J. Spika.** 1997. Persistence of *Escherichia coli* O157:H7 in dairy cattle and the dairy farm environment. Epidemiol. Infect. **119:**251–259.
45. **Renwick, S. A., J. B. Wilson, R. C. Clarke, H. Lior, A. A. Borczyk, J. Spika, K. Rahn, K. McFadden, A. Brouwer, A. Copps, G. Anderson, D. Alves, and M. A. Karmali.** 1993. Evidence of direct transmission of *Escherichia coli* O157:H7 infection between calves and a human. J. Infect. Dis. **168:**792–793.
46. **Rice, D. H., D. D. Hancock, and T. E. Besser.** 1995. Verotoxigenic *E. coli*

O157 colonisation of wild deer and range cattle. Vet. Rec. **137:**524.
47. **Riley, L. W., R. S. Remis, S. Helgerson, H. B. McGee, J. Wells, B. Davis, R. J. Hebert, E. S. Olcott, L. Johnson, N. Hargrett, P. A. Blake, and M. L. Cohen.** 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N. Engl. J. Med. **308:**681–685.
48. **Samadpour, M., J. E. Ongerth, J. Liston, N. Tran, D. Nguyen, T. S. Whittam, R. A. Wilson, and P. I. Tarr.** 1994. Occurrence of Shiga-like toxin-producing *Escherichia coli* in retail fresh seafood, beef, lamb, pork, and poultry from grocery stores in Seattle, Washington. Appl. Environ. Microbiol. **60:**1038–1040.
49. **Sanderson, M. W., J. M. Gay, T. E. Besser, D. D. Hancock, L. K. Fox, and C. C. Gay.** 1995. Sensitivity of bacteriologic culture for detection of *Escherichia coli* O157:H7 in bovine feces. J. Clin. Microbiol. **33:**2616–2619.
50. **Strauch, D.** 1991. Livestock manure as a vector for infectious agents. Dtsch. Tieraerztl. Wochenschr. **98:**265–268.
51. **Strauch, D.** 1991. Survival of pathogenic micro-organisms and parasites in excreta, manure and sewage sludge. Rev. Sci. Tech. O. I. E. (Off. Int. Epizoot.) **10:**813–846.
52. **Su, C. Y., and L. J. Brandt.** 1995. *Escherichia coli* O157:H7 infection in humans. Ann. Intern. Med. **123:**698–714.
53. **Tarr, P. I.** 1995. *Escherichia coli* O157:H7: clinical, diagnostic, and epidemiological aspects of human infection. Clin. Infect. Dis. **20:**1–10.
54. **Tarr, P. I., and M. A. Neill.** 1996. The problem of non-O157:H7 Shiga toxin (verocytotoxin)-producing *Escherichia coli*. J. Infect. Dis. **174:**1136–1139.
55. **Tauxe, R. V.** 1997. Emerging foodborne diseases: an evolving public health challenge. Emerg. Infect. Dis. **3:**425–434.
56. **Trevena, W. B.** 1997. Associations between human infection with Vero cytotoxin-producing *Escherichia coli* O157 and farm animal contact, abstr. no. V28/I, p. 3. *In* VTEC'97, 3rd International Symposium and Workshop on Shiga Toxin (Verocytotoxin)-Producing *Escherichia coli* Infections.
57. **U.S. Department of Agriculture Animal and Plant Health Inspection Service.** 1996. Prevalence of verotoxic *Escherichia coli* O157:H7 in lambs at slaughter, p. 1–5. *In* APHIS report of FY 1995 accomplishments in animal production food safety. Veterinary Services, Englewood, Colo.
58. **U.S. Department of Agriculture Animal and Plant Health Inspection Service.** 1997. An update: *Escherichia coli* O157:H7 in humans and cattle, p. 1–28. *In* Report from Centers for Epidemiology and Animal Health. Centers for Epidemiology and Animal Health, Fort Collins, Colo.
59. **Wallace, J. S., T. Cheasty, and K. Jones.** 1997. Isolation of Vero cytotoxin-producing *Escherichia coli* O157 from wild birds. J. Appl. Microbiol. **82:**399–404.
60. **Wang, G., T. Zhao, and M. P. Doyle.** 1996. Fate of enterohemorrhagic *Escherichia coli* O157:H7 in bovine feces. Appl. Environ. Microbiol. **62:**2567–2570.
61. **Waters, J. R., J. C. M. Sharp, and V. J. Dev.** 1994. Infection caused by *Escherichia coli* O157:H7 in Alberta, Canada, and in Scotland: a five-year review, 1987–1991. Clin. Infect. Dis. **19:**834–843.
62. **Weinstein, D. L., M. P. Jackson, L. P. Perera, R. K. Holmes, and A. D. O'Brien.** 1989. In vivo formation of hybrid toxins comprising Shiga toxin and the Shiga-like toxins and the role of the B subunit in localization and cytotoxic activity. Infect. Immun. **57:**3743–3750.
63. **Wells, J. G., L. D. Shipman, K. D. Greene, E. G. Sowers, J. H. Green, D. N. Cameron, F. P. Downes, M. L. Martin, P. M. Griffin, S. M. Ostroff, M. E. Potter, R. V. Tauxe, and I. K. Wachsmuth.** 1991. Isolation of *Escherichia coli* serotype O157:H7 and other Shiga-like-toxin-producing *E. coli* from dairy cattle. J. Clin. Microbiol. **29:**985–989.
64. **Woolcock, J. B.** 1991. Microbiology of animals and animal products. Elsevier Science Publishing Company, Inc., New York, N.Y.
65. **Zhao, T., M. P. Doyle, J. Shere, and L. Garber.** 1995. Prevalence of enterohemorrhagic *Escherichia coli* O157:H7 in a survey of dairy herds. Appl. Environ. Microbiol. **61:**1290–1293.